BioRisk 6: 41–60 (2011)
doi: 10.3897/biorisk.6.1568
www.pensoft.net/journals/biorisk

RESEARCH ARTICLE

A peer-reviewed open-access journal
BioRisk

A strategy for conservation and investigation of the protected resurrection plant *Haberlea rhodopensis* Friv.

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Academic editor: *J. Settele* | Received 17 May 2011 | Accepted 1 November 2011 | Published 19 December 2011

Citation: Daskalova E, Dontcheva S, Yahoubian G, Minkov I, Toneva V (2011) A strategy for conservation and investigation of the protected resurection plant *Haberlea rhodopensis* Friv. BioRisk 6: 41–60. doi: 10.3897/biorisk.6.1568

Abstract

Representatives of the resurrection plants from *Gesneriaceae* family are included in the Red Book of Bulgaria, in the European Register of rare, endangered, and endemic plants, and are subjects of world's conventions on the preservation of the biodiversity. The unique feature of these plants to recover from prolonged dehydration (anabiosis) is explored in numerous studies. These species are also Tertiary relics, so they could give us important knowledge about plant evolution.

Our research group at the University of Plovdiv has established a national *in vitro* gene bank for *Haberlea rhodopensis* Friv. (25 localities) and *Ramonda serbica* Panc. (2 localities) from Bulgaria. The national gene bank is based on original and modified *in vitro* technologies and can serve as a conservation and biodiversity investigation center for the family *Gesneriaceae*.

Basing on our work with *Haberlea rhodopensis* Friv., we are developing a **strategy for conservation and investigation of rare and relic plant species** (mapping and exploration of habitats – assessing the local risk of extinction - introducing in an *in vitro* gene bank - model plants for research – adaptation and possible re-introduction in endangered habitats). This strategy can be adapted and used for conservation and investigation of other rare, protected, relic and endemic plants from other regions of Europe and worldwide.

Keywords

Haberlea rhodopensis Friv., risk assesment scoring system, *in vitro* gene bank

Introduction

Bulgaria is a country with rich biological diversity. The registered so far Bulgarian and Balkan endemic flowering plants are about 170 species and 100 subspecies. Such abounding

biodiversity can be explained with many factors and the most important of them is the geographical position of the country, the diversity of climate zones and the special geological history of the Balkans. During the ice age, the climate in the Balkan Peninsula was milder and the glaciations were weaker than in the other parts of Europe. Perhaps this is one of the reasons why so many relic and endemic plants had survived in the Balkans, but not in other locations of Europe (Huttunen et al. 1992; Velev 2002). These ancient plants could be a source of valuable functional and evolutionary information.

At present, plant biodiversity in the country is endangered by a number of negative factors, most of them of antropogenic nature. Global climatic changes also lead to negative consequences on natural ecosystems (Peev and Delcheva 2007).

Among the especially interesting plants are the Balkan representatives of the family *Gesneriaceae* which includes over of 3000 species and 140 genera, most of them are tropical plants (Kubitzki and Kadereit 2004). On the territory of Bulgaria, there are two species of the family *Gesneriaceae*: *Haberlea rhodopensis* Friv. (Rhodope silivriak, Orpheus' flower) and *Ramonda serbica* Panč (Serbian Ramonda).

These Balkan *Gesneriaceae* species are termed as resurrected plants because of their unique ability to survive almost complete dehydration with minimum metabolic activity (anabiosis). After being rehydrated, they quickly return to normal physiological condition. With their ability to keep about 80% of the chlorophyll contents at the time of anabiosis, these plants have become a model system for studying the photosynthetic and metabolic processes, as well as physiological stability when exposed to prolonged drought (31- month recovery was recorded for *H. rhodopensis*) (Kimenov et al. 1974, Markovska et al. 1994).

The resurrected plants of the family *Gesneriaceae* have been a subject of long-lasting scientific interest in Bulgaria, Europe, China, USA, Canada and many other countries (Stefanov et al. 1992; Markovska et al. 1994; Muler et al. 1997; Georgieva et al. 2007; Mihailova et al. 2011).

Our research is concentrated mainly on *Haberlea rhodopensis* Friv.

The Rhodope "silivriak" is a perennial herbaceous plant with purple flowers and leaves in a rosette. The plant is a typical chasmophyte. It grows on limestone or silicate rocks, mostly in rock rifts in overshadowed places with high air humidity, in beech and pine tree forests. The reproduction is vegetative and sexual. It flourishes in May-July; the fruits and seeds become ripe in July – August. (Stojanov et al. 1966; Velchev et al. 1975).

The plant is endemic to Bulgaria and Greece and is one of the relict elements of the tertiary times. It can be found in the Central Balkan mountains, Balkan foothills, Northern, Eastern and Central Rhodopi mountains in Bulgaria, and in northeastern Greece (Menikion, Pangeon, Falakron, Rodopi, Papikion and the Nestos river gorge) (Bazos and Petrova 2010).

There are some differences between the global and regional conservation statuses of *Haberlea rhodopensis* Friv. The species is included in Bulgaria's Red Book in the three categories: Rare species, Balkan endemic species, and Tertiary relic. In Bulgaria, collecting of the plant from its natural habitats is prohibited except if it is done with a special permission order (The Committee of Nature Conservation at the Ministry of Bulgaria, 1984). The plant is also listed in the European Register of rare, endangered, and endemic plants under the Rare Species category (Committee for Conservation of Nature at the Ministry of Bulgaria 1984; Peev and Delcheva 2007). *Haberlea rhodopensis* is listed in Appendix I of the Convention of European Wildlife Conservation and Natural Habitats (Bern Convention). Together with *Ramonda* sp., the species is a subject of world's conventions on the preservation of the biodiversity representing the resurrecting plants of the Northern Hemisphere. In Greece, *Haberlea rhodopensis* is protected under the Greek Law Presidential Degree 67/81 and can be found in five "Natura 2000" sites. It is listed as Vulnerable in Greek Red Book . Nevertheless, the plant is listed as Least Concern (LC) on the (IUCN) Red List of Threatened Species (Petrova and Vladimirov 2009; Bazos and Petrova 2010).

As part of a conservation project, our research group has created a national genetic bank *in vitro* at the University of Plovdiv. It contains *Haberlea* plants from various habitats in Bulgaria, covering all Bulgarian populations. The genetic bank is maintained using original *in vitro* technologies (Jungnickel et al. 1992; Tóth et al. 2004; Djilianov et al. 2005), modified and adapted for *Haberlea* by our research group (Dontcheva et al. 2009). These include dry sterilization of seeds, micropropagation (multiplication) to the desired number of plants, and adaptation).

Unlike other conservation methods (seed banks, botanic garden collections, etc.), the in vitro gene bank contains living plants that can be continuously propagated and then used for research and conservation purposes and there will be no need to collect them from the natural habitats. As a donor of plant material, the in vitro gene bank has the following advantages:

- Year-round access to plants of various source populations and at different developmental stages.
- For certain experiments, genetically identical in vitro plants (clones) could be selected, and it can guarantee monofactor experiments.
- Possibility for selection of plants with desired constant characteristics (drought resistance, antioxidant potential, antimicrobial activity). The selected plants can then be multiplied to numbers necessary for analyses.
- Possibility to grow elite, pathogen-free plants on small area, under completely controlled uniform conditions (temperature, light, nutrient medium).

In our conservation efforts, the development of ***in vitro* gene bank** is a central part of a broader approach. Basing on our experience with *Haberlea Rhodopensis* Friv., we are developing a **strategy for conservation and investigation of rare and relic plant species** (mapping and exploration of habitats - assessing the risk of extinction, intro-

ducing in an *in vitro* gene bankmodel plants for research and adaptation – possible re-introduction in endangered habitats).

Another important part of the strategy is the elaboration of local-scale **risk assessment scoring system** for *Haberlea rhodopensis* Friv., which is used to evaluate the risk of extinction of species from any particular locality of its habitat. The local risk assessment scoring is based on two-year monitoring of 20 representative localities, covering all Bulgarian *Haberlea* populations which includes GPS recording of spatial data as well as documentation of morphology and phenology traits and ecology of the species. The risk assessment results are the basis for the decisions in the last phase of the strategy - **the re-introduction** of adapted *in vitro* plants back to the endangered localities. This local scale scoring system can also be used for future re-assessment and re-evaluation of the status of *Haberlea rhodopensis* in the global risk assessment systems, such as IUCN Red List *of Threatened Species* (Mace et al. 2008).

The conservation and investigation strategy can be adapted and used for other rare, protected, relic and endemic plants from other regions of Europe and worldwide.

Methods

The strategy for conservation and investigation of the protected and resurrection plant *Haberlea rhodopensis* Friv. includes the following steps:

1. GPS mapping and exploration of *Haberlea* localities in combination with monitoring of some characteristics of plant biology (phenology, morphology, anabiosis, age structure, etc.);
2. Development of risk assessment criteria for evaluation of the risk of extinction for particular *Haberlea* localities;
3. Seeds collection and introduction of *Haberlea* plants from various habitats in the in vitro gene bank;
4. Micropropagation and multiplication of *Haberlea* plants for research, adaptation and re-introduction;
5. Adaptation and re-introduction of some representatives from localities with high risk of extinction in their natural endangered habitats.

Habitat exploration and risk assessment

The observations made in Item 1 give the basis for Item 2 (the risk assessment table for *Haberlea rhodopensis*).

Using GPS mapping, spatial data for each *Haberlea* locality (previously known or newly discovered) were recorded. A GPS navigation apparatus was used, model Garmin® Oregon 400t. The neighbouring localities were mapped as separate ones if the distance between them was at least 1 km, otherwise they were counted as a single locality.

For each locality, the following spatial parameters were recorded:
- Area (m^2)
- Altitude (m)
- Orientation (according to the geographic directioins – N,E,S,W or intermediate)
- Isolation (distance from the nearest locality (in km)

As another feature of spatial localization we also documented the accessibility to the plants, e.g. if they grow on high steep rocks, near or far from popular tourist routes, in remote and desolate places that may increase the risk of picking up by tourists; we also recorded the position of each locality when it is in a protected area, the type (degree) of protection. Most of the recorded spatial parameters are used in the first module of the Risk assessment scoring table.

During the expeditions, certain physiological, phenological and ecological characteristics were monitored for twenty localities. These more intensely monitored localities were chosen as representative because each of them is either unique in some aspects, or shows typical characteristics for a neighboring group of 2-5 localities. The representative localities are described in Table 1.

The monitored morphology and phenology characteristics were the following:
- Average plant density (number of rosettes per m^2)
- Average leaf size
- Changes in leaf shape and color
- Changes in flower size and morphology
- Time of flowering
- Time of seed production
- Presense of drought-induced (summer) anabiosis stage
- Time of entering anabiosis stage
- Duration of anabiosis stage
- Time of recovery from anabiosis stage
- Presence of frost-induced (winter) anabiosis stage

The average plant density was calculated as follows: for most localities, five square spots 1×1 m were chosen, in which the plant rosettes were counted and the average number was recorded. In small localities, all the plants in the locality were counted and the number was divided by the total locality area in m^2, measured with the GPS device.

The average leaf size for a locality was recorded after measuring and averaging the length of the central vein of 50 leaves of adult (vegetative and generative) *H. rhodopensis* plants, without tearing the leaves from the rosettes or harming them.

For the representative localities, the age structure was also monitored. The number of individuals at different ontogeny stages (Figure 3) was recorded using the square spots previously mentioned. It was recorded whether some of the age categories were missing, which could be a sign for disturbed population structure and possible risk indicator.

Table 1. Spatial parameters of 20 representative localities of *H. rhodopensis*

No	Representative Locality	Population/ subpopulation	Coordinates	Altitude	Area, m^2	Orientation
1	Madzharovo	East Rhodopean	N41°38.510 E025°52.760	135±10m	280±10	N
2	Studen Kladenetz	East Rhodopean	N41°36.934 E025°39.854	170±15	30±5	N
3	Bezvodno	East Rhodopean	N41°45.201 E025°05.443	900-950±15	550 ±15	N,NE
4	Planinsko	Central Rhod. (Eastern)	N41°46.474 E024°57.272	1520±15	350 ±30	N,NE
5	Belitza	Central Rhod. (Eastern)	N41°49.801 E024°52.896	680±15	240 ±10	N,NW, NE
6	Bachkovo	Central Rhod. (Eastern)	N41°55.698 E024°51.003	520-650 ±15	2300 ±30	N,NW, NE
7	Gela	Gela	N41°38.741 E024°34.039	1450 ±10	50 ±10	NE
8	Smolyan, Ustovo quarter	Smolyan	N41° 34.432 E024°46.597	830 ±10	120±10	N
9	Smilian	Smolyan	N41°30.325 E024°46.450	770 ±10	85±10	NE
10	Gorna Arda	Smolyan	N41°26.886, E024°36.295	1140±15	55±10	N
11	Orehovo	Central Rhod. (Western)	N41°52.231 E024°36.171	940 ±15	1000 ±20	N,NE, NW, W,E, SE
12	Sitovo	Central Rhod. (Western)	N41°54.957 E024°36.250	1460±15	680±50	N,NE
13	Ustina	North Rhodopean	N42°02.092 E024°31.536	450 ±15	120 ±20	N
14	Skobelevo	North rhodopean	N42°00.173 E024°32.432	1150±15	450±50	NE,E
15	Trigrad	West Rhodopean	N41°36.693 E024°22.998	1100-1280 20±15	2500±50	N,NW, NE
16	Yagodina	West Rhodopean	N41°37.542 E024°21.962	1170 ±15	250±15	N
17	Lovetch	Balkan Foothills	N43°07.271 E024°42.241	380 ±15	260±20	N,NW
18	Troyan	North Balkan	N42°47.934 E024°40.507	750 ±10	850±30	N,NE
19	Byala reka, Kalofer	South Balkan	N42°37.816 E024°56.832	610 ±10	2000±50	N,NW, NE
20	Tazha	South Balkan	N42°40.011 E025°02.842	550 ±15	1300±20	N,NE,E

Each locality in the table has the same name as the name of the nearest town/village

When possible/available, ecology factors were also recorded for some of the representative localities, including:

- Abiotic factors (drought, light exposure, rock type, etc.)
- Biotic factors (associated plant species, pathogens, parasites, pollinators, etc.)
- Anthropogenic factors (pollution, urbanization, etc.)

All observations and measurements were performed during the expeditions done in two consecutive years (2009 and 2010), 3 times each year for the representative localities: in spring (April-June), summer (July-August) and autumn (October-November) in order to cover the most important seasonal changes. Summer expeditions were combined with collection of seeds for *in vitro* micropropagation.

Selected characteristics were used for elaborating the risk assessment scoring table. They are applicable at local scale for evaluating the risk of extinction for each particular *Haberlea* locality and were used for completing the **Risk assessment scoring table** for *Haberlea rhodopensis* Friv.

The in vitro gene bank

The following two steps preceded the introduction of *Haberlea* plants in *in vitro* culture:

- **Seeds Collection.** The seeds were collected in July during expeditions to the habitats of interest in the Rhodopes and the Balkan Mountains. For better conservation, the seeds were collected together with seed capsules and stored in a dry and airy place at temperature near 25°C. The aim was to collect seeds from as much plants as possible, covering the entire reachable area of the locality. This approach allows maximum of the habitat biodiversity to be included into the *in vitro* gene bank. In order to not hamper the natural reproduction of plants, not more than 1–2 capsules from each plant (on average) were collected.
- **Germination test.** The seed capsules were removed, and the very fine seeds (under 0.5 mm in diameter) were passed through fine sieves. In order to test the viability of the seeds in vivo, they were put on Petri dishes with moist filter paper and stored at 23°C until a plantlet with two green leaves appears.

By applying a direct organogenesis method (Tóth et al. 2004; Jungnickel et al. 1992; Djilianov et al. 2005),we have developed a modified in vitro micropropagation system. The method included the following stages:

- **Dry Sterilization.** The seeds were put into Eppendorf tubes and then sterilized in an exicator (excecator) on the fume of sodium hypochlorite and HCl.
- ***In vitro* germination**. Sterilized seeds were put in vitro in vials with nutrient medium WPM, without sugar and hormones. A normal organogenesis of individual microplants was observed at the end of this stage.
- **Micropropagation**. Haberlea rhodopensis microplants from the previous stage were transferred in nutrient media WPM with phytohormones BAP and IAA.

The medium was supplemented with an antioxidant - 200mg/l filter sterilized glutathione and the pH was stabilized with K-phosphate buffer.

- **Rooting**. In the end of this stage, the microplants formed a stable root system.
- **Adaptation**. The rooted plants were planted in vivo in a turf-perlite mixture 1:1 (v/v) and they were grown under controlled air humidity and temperature in a growth camera for three weeks. At the stages 2, 3 and 4 the microplants were grown in vitro in growth cameras with controlled temperature 21°C ± 1°C, at light regime 16 h light (day)/8 h darkness (night).

Results

Habitat exploration

In 2009 and 2010, over 80 expeditions were organized in mountain regions of Bulgaria (the Balkan, the Rhodopes). **Sixty seven (67) *Haberlea rhodopensis* localities** were mapped with GPS. The localities were conventionally divided into nine populations (some of them could be actually sub-populations) (Figure 1).

The twenty representative localities are the main subjects of monitoring. Each of them is either characteristic for a neighboring group of 2-5 localities, or it has some unique distinguishing features. For example, the locality Smolian/Ustovo is the only one discovered so far in which *H. rhodopensis* grows in urban conditions; the population Madzharovo has the lowest altitude (136 m) and it is the most remote Rhodpean locality (26 km to the nearest locality Studen kladenetz)

Some of the spatial characteristics of the representative localities are shown in Table 1.

67 mapped localities cover about 95% of the known distribution area of *Haberlea rhodopensis* in Bulgaria. About 20% of the localities are new (discovered for the first time during our expeditions). Thorough GPS mapping of the *Haberlea* localities has been performed for the first time in Bulgaria by our research group.

The GPS coordinates, altitude, orientation, distance to the nearest locality and area of all localities were recorded. Additional seasonal monitoring and exploration were performed for the representative localities, as described in Methods.

The area of the localities varies widely - from several m^2 to more than 3 km^2. The distance from the nearest locality also varies widely – from 1 to more than 20 km.

Morphology, phenology, anabiosis, and ecology

During the expeditions, some major variations in *Haberlea rhodopensis* population density and morphology traits were observed. Almost all morphological characteristics, phenological traits, as well as the presence and duration of the anabiosis stage vary significantly between the localities. Examples of such extreme differences are given in Figure 2 and Table 2.

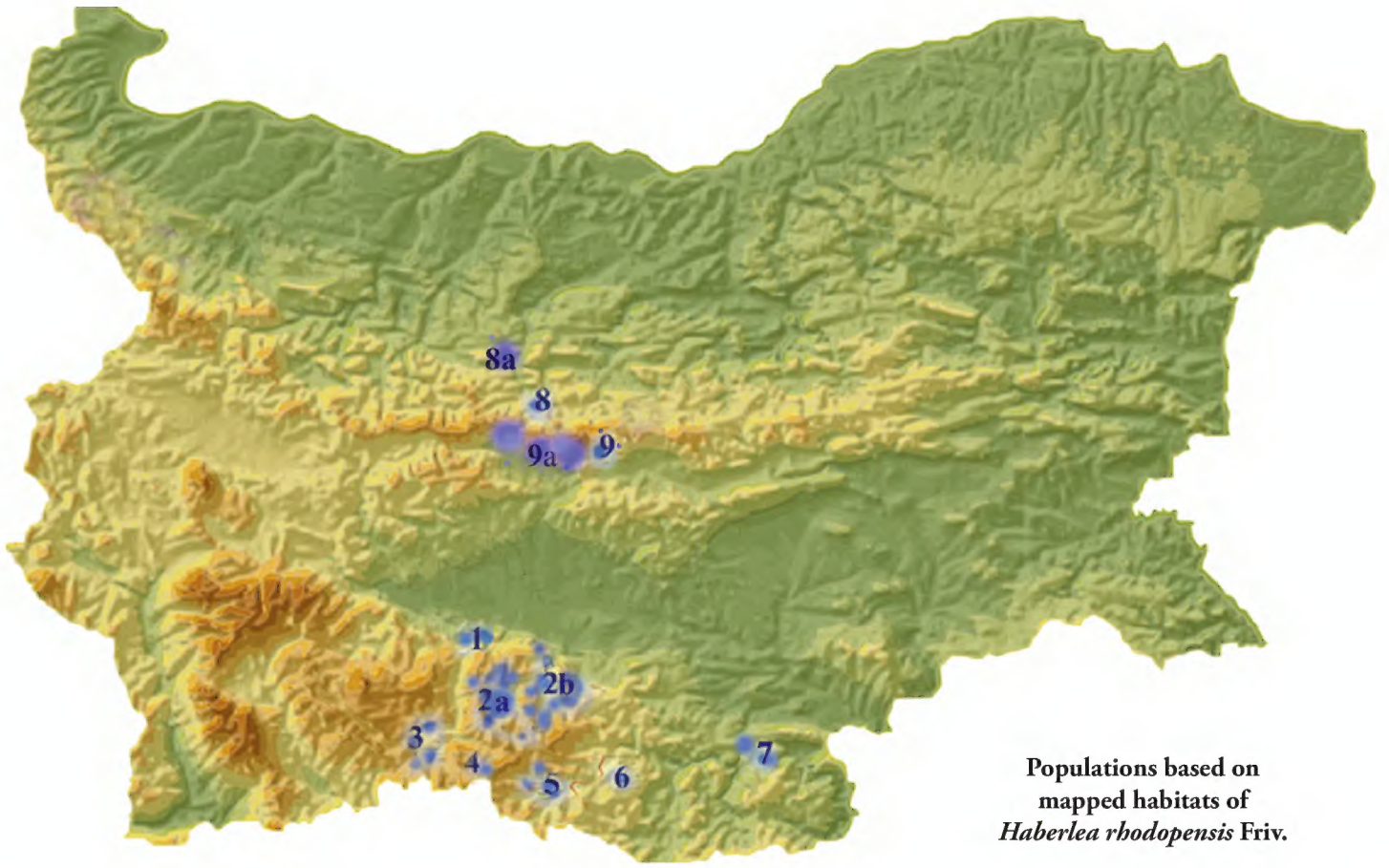

Figure 1. GPS-mapped localities of *Haberlea rhodopensis* Friv. The localities group into the following populations/subpopulations: 1 - North Rhodopean **(4);** 2a - Central Rhodopean (Western) **(30);** 2b - Central Rhodopean (Eastern) **(5);** 3 - West Rhodopean **(8);** 4 - Gela and surrounding regions **(1);** 5 - Smolyan and surrounding regions **(8);** 6 - East Rhodopean 1 (Ardino and surrounding regions) **(2);** 7 - East Rhodopean 2 (Madjarovo-Studen Kladenetz) **(2);** 8a - Balkan Foothills (Lovetch and surrounding regions **(1);** 8b - North Balkan (Troyan and surrounding regions) **(1);** 9 - South Balkan **(5).** The number in **bold** in brackets represents the number of mapped localities in the population/subpopulation.

Age structure

The age structure was monitored for the representative localities. Due to the fact that it is not possible to detect the real age of each individual plant, the population structure analysis was based on the number of individuals in the different ontogenetic stages of the life cycle. Four age (stage) categories were observed for *Haberlea rhodopensis* (Figure 3).

It was recorded for each representative locality whether all age categories were present. Missing age categories could indicate disturbed population structure.

Ecology

When possible, some ecology factors were also recorded for the representative localities, including:

- Abiotic impact (drought, light exposure, rock type, etc.)
- Biotic impact (associated plant species, pathogens, parasites, pollinators, etc.)
- Anthropogenic impact (pollution, urbanization, etc.)

Figure 2. Extreme differences in plant density, leaf size and phenology exemplified with four representative localities: **A** "Studen kladenetz". The plants in this locality are characterized with early anabiosis (end of April) and disturbed population structure (lack of young and predominating ageing individuals). **B** "Gradishteto", near Skobelevo village. Several unusual localities like this are positioned on rocks exposed to direct sunlight and severe drought; nevertheless they show stable population structure. They also have many morphology and phenology changes compared to localities like **C** and **D**. **C** "Uzunov kamak" near Gorna Arda village. In this area, there are many potetntial habitats of *H. rhodopensis*, nevertheless the species is rare and the localities are isolated from one another with more than 5 km. **D** "Eremliitza", near Sitovo village. The population is stable, the conditions are beneficial. The locality is positioned in an area with many neighboring localities.

At the end of each year of monitoring the seasonal observations were summarized and the scoring table was filled in for each of the representative localities. There were no significant differences observed in spatial, morphology and phenology parameters of the localities between the two years (2009 and 2010). Some changes in the age structure were observed in six localities: an increase of ageing individuals in "Studen kladenetz" and "Ustina" localities, and an increase of young individuals (plantlets with 2-6 leaves) and vegetative adults in "Bachkovo" "Sitovo", "Smilian" and "Planinsko" localities. The number of generative adults (flowering and seed producing individuals) remains relatively stable in all localities.

Biodiversity risk assessment scoring system

The risk assessment scoring system for *Haberlea rhodopensis* Friv. is based on monitoring and evaluation of potential risk factors for the localities. The risk assessment criteria are divided in 6 modules, including 5 factor modules and one combinatory module

Table 2. Differences in spatial parameters, plant density, leaf size and phenology traits in 4 representative localities, as shown in Figure 2: **Locality 1** - "Studen kladenetz", near Studen kladenetz village; **Locality 2** - "Gradishteto", near Skobelevo village; **Locality 3** "Uzunov kamak" near Gorna Arda village; **Locality 4** "Eremliitza", near Sitovo village.

Parameter	Locality 1	Locality 2	Locality 3	Locality 4
Area (m^2)	30±5	240±15	55±10	480±50
Altitude	170±15 m	1150 ± 20 m	1140±15 m	1460±15 m
Average number of rosettes per m^2	45	58	23	32
Average leaf size, mm	36	32	72	78
Time of flowering	April 3rd decade	May 2nd decade	June 1st decade	June 2nd decade
Time of seed production	not detected	July 3rd decade	August 3rd decade	September 2nd decade
Time of entering anabiosis stage	April 3rd decade	July	August	no anabiosis state
Duration of anabiosis stage	6 months	3 months	1 month	no anabiosis state

that accounts for combinatory impacts (interactions between factors). The "Anabiosis" module is unique for the resurrection plants.

The risk assessment scoring system was elaborated as a question form with positive (Y) or negative (N) possible answers (Table 3). The absolute values (from 0 to 5) express/show the relative importance of the particular factor. If the value is zero, the corresponding Yes or No answer is either non-informative or not important for the risk assessment. The negative signs of the values indicate negative influence (increased risk), while the positive ones are related to beneficial (risk-decreasing) influences of the relevant criterion. Thus, each question contributes to the total risk score for the locality (R) which is calculated as a sum of all values of "Y" and "N" columns.

The question mark and the "x" values in the last column of the Risk scoring table indicate the degree of uncertainity – the questions whose answers are not clear for the particular localities. If the answer of the particular question is not known ("x" in the column "?"), the corresponding "Yes" and "No" cells on the same row are filled with a zero value. For each locality, the "x" marks are counted. The more x-es, the more uncertain the risk scores for the particular locality are.

We applied the local risk assessment system to calculate the risk scores (R) and uncertainity (x) for the 20 representative localities. The top 3 most endangered localities are:

1. Studen Kladenetz, East Rhodopean population (R=-39, x=4)
2. Ustina, North Rhodopean population (R=-38, x=3)
3. Madzharovo, East Rhodopean population (R=-33, x=4).

Generally, the most endangered population is the East Rhodopean one, followed by North-Rhodopean. The East Rhodopean habitats of *Haberlea rhodopensis* are strongly

Table 3. Risk assessment scoring system for *H. rhodopensis*

Q	Criterion	Y	N	?
	Module 1: Spatial parameters			
1.01	**Locality area (choose one of the following)**			
1.01A	The locality area is less than 10m^2	-5	0	X
1.01B	The locality area is between 20 and 100 m^2	-2	0	X
1.01C	The locality area is between 100 and 300 m^2	0	+2	X
1.01D	The locality area is more than 300 m^2	0	+4	X
1.02	**Altitude (choose)**			
1.02A	The altitude of the locality is below 400 m a.s.l.	-3	0	X
1.02B	The altitude of the locality is between 500 and 1800 m a.s.l.	0	+3	X
1.02C	The altitude of the locality is higher than 1800 m a.s.l.	-1	0	X
1.03	**Isolation (choose)**			
1.03A	The distance from the nearest locality is 1-3 km	0	+3	X
1.03B	The distance from the nearest locality is 4-10 km	-2	+1	X
1.03C	The distance from the nearest locality is more than 10 km	-3	0	X
1.04.	**Accessibility**			
1.04A	The locality is near popular tourist routes or is easily accessible by tourists	-2	+2	X
1.05	**Protection (choose)**			
1.05A	The locality is positioned in sustained reserve	+2	-1	X
1.05B	The locality is positioned in national park	+1	0	X
1.05C	The locality is not positioned in protected area	-1	+1	X
	Module 2: Morphology, physiology and phenology			
2.01.	**Average plant density (choose)**			
2.01A	The number of rosettes is less than 3 per m^2	-2	0	X
2.01B	The number of rosettes is between 5 and 30 per m^2	+3	-1	X
2.01C	The number of rosettes is more than 40 per m^2	-1	0	X
2.02	**Leaf and flower morphology (all)**			
2.02A	The leaves of adult plants are unusually small	-2	+1	X
2.02B	The leaves have unusual shape	-1	+1	X
2.02C	The leaves have unusual colour (e.g.yellow)	-2	+1	X
2.02D	The flowers have unusual morphology	-1	+1	X
2.03	**Phenology (all)**			
2.03.1	The flowering is in May-June (depending on altitude)	+2	-2	X
2.03.2	The seeds ripe in July-August (depending on altitude)	+2	-2	X
	Module 3. Anabiosis			
3.01	**General questions (All)**			
3.01.1	The plants in the locality get into drought-induced anabiosis	+1	+2	X
3.01.2	The plants in the locality get into frost-induced anabiosis	+1	0	X
3.02	**Time of entering anabiosis (choose)**			
3.02A	The plants enter drought-induced anabiosis in July-August	+2	0	X
3.02B	The plants enter drought-induced anabiosis in May-July	-3	0	X
3.03	**Time of recovery from anabiosis (choose)**			
3.03A	The plants recover from drought anabiosis in October-November	+2	-1	X
3.03B	The plants recover from drought anabiosis in the next year spring	+1	0	X

Q	Criterion	Y	N	?
3.03C	No observed recovery from drought anabiosis	-5	+2	X
	Module 4. Age structure (all)			
4.01	All age categories are present in the locality	+4	-1	X
4.02	Young plants predominate	+2	0	X
4.03	Young plants missing	-3	+1	X
4.04	Vegetative adults predominate (vegetative reproduction only)	-1	0	X
4.05	Generative adults predominate (vegetative+sexual reproduction)	+3	0	X
4.06	Generative adults missing	-3	0	X
4.07	Ageing plants predominate	-4	+1	X
	Module 5. Ecology (all)			
5.01	Negative abiotic impacts on the locality (drought, light exposure, etc.)	-3	+1	X
5.02	Positive abiotic impacts on the locality	+3	-1	X
5.03	Negative biotic impacts (pathogens, parasites invasive associated plants etc.)	-3	+1	X
5.04	Positive biotic impacts (beneficial plant associations, pollinators etc)	+3	-1	X
5.05	Negative anthropogenic impacts (pollution, urbanization etc)	-3	+1	X
5.06	Positive Anthropogenic impacts (measures for protection etc)	+2	-1	X
	Module 6. Combinatory impacts			
6.01	**Combined spatial parameters**			
6.01A	Positive answer to questions 1.01A,1.02A and 1.03C	-5	+1	X
6.01B	Positive answer to questions 1.01C or D, 1.02B and 1.03A	+5	0	X
6.01C	Positive answer to questions 1.01B, 1.03B and 1.05C	-2	0	X
6.02	**Other combinations**			
6.02A	Positive answer to any variants of question 2.02, and to questions 3.02B, 3.03C,4.03, 4.07	-5	+1	X
6.02B	Positive answers to questions 3.02A, 3.03A, 4.01 or 4.02 or 4.05	+4	0	X
6.02C	Positive answers to questions 5.01 or 5.03 or 5.05, and negative answers to 5.02 or 5.04 or 5.06	-4	0	X
6.02D	Negative answers to questions 5.01 or 5.03 or 5.05, and positive answers to 5.02 or 5.04 or 5.06	+4	0	X
	Total risk score for the locality (R):			

isolated (more than 10 km far from each other), which increases the risk of inbreeding and decreasing the natural diversity. The high temperatures and dry conditions cause early entering an anabiotic state (Figure 2A). The age structure is disturbed, often with predomination of ageing colonies and lack of plantlets and young plants.

Modified in vitro micropropagation system for *Haberlea rhodopensis* Friv.

- **The in vitro gene bank**

The method of dry sterilization of seeds has been applied for the first time to the family *Gesneriaceae*, which is an original contribution of our research team. During the micropropagation stage, the microplants grow in a medium with phytohormones and an antioxidant added. As a result, a buldge containing multiple microplants is formed,

Figure 3. Age (stage) categories of *H. rhodopensis:* **A** Young single plants with 1-6 leaves, 1-3 months after growing from seeds; **B** Vegetative adults that reproduce vegetatively and form colonies, but do not reproduce sexually; **C** Generative adults (flowering and seed producing individuals); **D** Ageing individuals with signs of necrosis and/or irreversible dessication.

which are then separated under sterile conditions in a laminar box. Thus, the micro-plants can be multiplied until the number required is reached. Some of the plants are stored as a live collection of in vitro plants from different habitats, while other can be used as model plants for multidisciplinary research and education.

During adaptation, the rooted plants are planted in vivo in a turf-perlite mixture and grown under controlled air humidity and temperature. Our team has achieved successful adaptation of *Haberlea rhodopensis* Friv. at the Plant Biotechnology laboratory of the University of Plovdiv which is a pre-requisite for successful reintroduction.

During the expeditions, seeds were collected from 39 localities, which cover all nine Bulgarian populations. The collected seeds were the basis for establishing the *in vitro* gene bank. At present, 25 localities (including almost all representative localities), were introduced in the gene bank and were grown as *in vitro* plants. The final goal is to establish and maintain a live in vitro collection of *Haberlea rhodopensis* plants, as representative for Bulgaria. In the future, the gene bank will cover also part of the Greek populations.

- **The re-introduction**

At this point of the development of our conservation approach, the re-introduction step has not been implemented yet. At this stage of the research the main goals are

to improve the risk assessment of the habitats, and to achieve the best possible representation of *H. rhodopensis* natural biodiversity in the *in vitro* gene bank. Regarding the re-introduction, the tendency will be to return in the wild in vitro plants that originate from the same locality in order to avoid risks of genetic swamping and outbreeding depression.

Populations for re-introduction will be chosen according to the following criteria:
(1) the risk of extinction of the species from a particular habitat;
(2) the threat of habitat destruction, and
(3) the importance of a particular habitat for the distributional range of *H. rhodopensis*.

Discussion

Haberlea rhodopensis: responses and variations

During the expeditions we observed some variations that are quite extreme in *Haberlea rhodopensis* morphology, plant density and phenology (Fig. 2). The ecological responses of the species are more diverse than expected. We can explain the observed differences mostly as a response to the variations in basic environmental conditions in the habitats. For example, in relation to the different light and water conditions, two types of *Haberlea rhodopensis* habitats can be distinguished:

- "**Classic" habitats** – on overshadowed rocks facing the North, within beech or pine forests, often immediately near water sources, with high air humidity and altitude in most cases above 500 m. a.s.l.
- **Arid habitats** – on rocks directly exposed to the sunlight, out of the forest coverage, with low air humidity and high light exposure

There are also intermediate habitats that combine some of the characteristics of classic and arid habitats. Some of the localities are very interesting where the two types of habitats are mixed.

Almost all morphological characteristics, phenological traits, as well as the presence and duration of the anabiotic stage vary significantly between the two types of habitats. Arid habitats are characterized with bigger plant density, much smaller individual plants (Figure 2B, Table 2), and paler color, which corresponds to less chlorophyll content (Daskalova, unpublished data). The flowering and seed ripening generally occur 1-2 months earlier than in the classic habitats when located approximately at the same altitude. The plants from arid habitats also enter anabiotic stage 1-3 months earlier than these in classic habitats, and the anabiosis duration is usually longer, while the plants from some of the classic habitats may not enter anabiosis at all.

In some habitats at high altitude, a frost-induced (winter) anabiosis was also observed (Daskalova et al. 2010). This phenomenon has not been completely explored yet.

The above observations gave us reason to conclude that the ecological plasticity of the species is significant, and it adapts successfully even in unfavourable conditions. In near future, we are planning to assess the levels of genetic polymorphism among

Haberlea populations and to try to understand if there is also a genetic basis for some of the most striking morphology and phenology differences.

The risk assessment approach

Assessing the extinction risk is a fundamental issue in conservation biology. The structure of a species is complicated and multi-level: individuals, demes, subpopulations, populations, metapopulations, subspecies. That's why it is important to develop risk assessment systems on different ecological scales.

There are many methods for categorization of endangered species in scientific literature. Some of them are results of long lasting efforts of large groups of experts, and they are widely used as international standards for species evaluation. Perhaps, the most widely used one is the risk assessment system applied in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (IUCN 2001 (ver.3.11) (Mace 2000; Mace et al. 2008). It includes the following categories of species: Extinct (EX), Extinct in the wild (EW), Critically endangered (CR), Endangered (EN), Vulnerable (VU), Near threatened (NT), Least concern (LC), Data deficient (DD), and Not evaluated (NE). A species is considered under threat if it meets a set of criteria with decreasing stringency for the categories Critically endangered (CR), Endangered (EN) or Vulnerable (VU). In the IUCN system, for CR, EN or VU categories, it is necessary a taxon to meet any one of these criteria to be qualified for listing at that level of threat.(IUCN 2001). In brief, the IUCN criteria for species under threat are:

A. Reduction in population size of: ≥ 90% (CR); ≥ 70% (EN) or ≥ 50% (VU) over the last 10 years or three generations;
B. Geographic range less than: 100 km^2(CR); 5000 km^2(EN), or 20,000 km^2(VU);
C. Declining population size of at least: 25% within one generation (CR); 20% within two generations (EN), or 10% within three generations (VU)
D. Population size estimated to number fewer than: 50 (CR), 250 (EN), or 1000 (VU) mature individuals;
E. Quantitative analysis showing the probability of extinction in the wild of at least: 50% within 10 years or three generations (CR); 20% within 20 years or five generations (EN), or 10% within 100 years (VU).

These criteria can be applied to any taxonomic unit at or below the species level. Compared to the IUCN system, our risk assessment scoring system is designed mostly for spatial scales exploration and mostly for plant species with fragmented areas/habitats/ranges. It describes well the differences between the localities and in some cases it reveals significant differences in risk scores for different localities. For instance, in general, although our assessments for most of the *Haberlea* populations agrees with the IUCN 2001 evaluation for the species as Least concern (LC) (i.e. not directly threat-

ened), there are populations and localities where the risk is significantly higher, as the most East Rhodopean localities are. Thus, the species as a whole is classified as Least Concern globally, however, there are really endangered points in its habitat within some regions. When the global category is not the same as the national or regional category, the cases like this are defined in the literature, for example in (Gärdenfors et al. 2001).

The time scale and the uncertainities related to it pose another problem to risk assessment studies (D'Elia and McCarthy 2010). In the IUCN system the time dimensions are quite well defined. The criteria are based mostly on observations on middle and long-term tendencies. Conversely, our scoring system is better adapted for moment "snapshots" of the risk status of the particular localities. A series of such time points ("snapshots") would outline a tendency (decline/increase in population size or area, etc.). So far, we have only two time points (for the years 2009 and 2010) which is not enough to make clear deductions for future tendencies. However, this two-year monitoring suggests that most of the populations and subpopulations are stable.

Another difficulty in elaborating the risk assessment scoring system for *Haberlea rhodopensis* was to distinguish which characteristic states represent natural variations of plant responses (e.g. modifications caused by light or drought in the arid habitats) and which are risk indicators. Such uncertainties caused by natural variability are common in ecological studies (Akçakaya et al. 2000; Lukey et al. 2010)

The anthropogenic impact was also hard to assess. Generally, basing on our monitoring, we can assume that currently *H. rhodopensis* populations are much less affected by direct human activities than by anabiotic risk factors, includingclimatic changes.

Some combinations of unfavorable factors may carry additional risk, especially when they interfere the life cycle of plants. In reality, almost all modules and risk criteria are logically interrelated. For example, the low altitude is almost always related to higher temperature and drought, higher anthropogenic impact and longer anabiosis stage. Also, as our observations showed, the unusually early anabiosis often interferes the plant reproduction: early entering into anabiosis could precede the pollination of flowers and hamper the seed production. Moreover, severe drought conditions and high temperatures decrease the survival of young plants, disturbing this way the population age structure. Volis et al. 2009 reported similar observations ().

At this stage of work, only a few of the possible factor combinations have been included in the risk assessment system. Further monitoring is needed for better understanding and scoring the risk factors and impacts.

Another difficult question is to what extent *H. rhodopensis*, a Tertiary relic species, suffers from the fragmentation of its habitat. The species has a high degree of habitat fragmentation (patchy distribution), which is characteristic for most relic species and it is obviously not a recent event. Much more likely, *H. rhodopensis* habitat was fragmented quite long ago. Our two-year monitoring also did not detect any loss of habitat, which could be another indicator that *Haberlea* populations are relatively stable. There is a high probability that this ancient species has developed genetic systems

and reproductive traits allowing it to overcome the effects of small population size and isolation (Falk and Holsinger 1991). On the other hand, the habitat fragmentation could have both positive and negative impacts on biodiversity (Fahrig 2003). For this reason, the genetic polymorphism among *Haberlea* populations and sub-populations will be main subject of future analyses of our research group. The *in vitro* gene bank will be used as all-year donor of high quality plant material for these analyses, as well as for re-introduction.

Conclusions

The described methods outline a strategy for conservation, research and sustainable development for *Haberlea rhodopensis,* a combination of field ecology and plant *in vitro* culture technology. In the future, it could be adapted and used for conservation and investigation of other rare, protected, relic and endemic plant species. We hope this research will help with the better understanding of genetics, biology, ecology and evolution of this unique species.

Acknowledgments

This research is supported by the National Science Fund, at the Ministry of Education and Science, Bulgaria, grant № DO 02/236.

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